V. DISCUSSION

5.1. Isolation of *A. hydrophila*

*A. hydrophila* is a ubiquitous Gram-negative, facultatively anaerobic bacterium of continental aquatic environments (Hazen *et al*., 1978; Popoff, 1984). These bacteria are mainly distributed in seawater, irrigation water, river water, brackish water, freshwater, groundwater, spring water, sewage-contaminated water, and activated sludge (Ashbolt *et al*., 1995; Borrel *et al*., 1998; Soler *et al*., 2002), both polluted and unpolluted fresh water and in association with aquatic animals. (Cahill, 1990; Stecchini and Domenis, 1994; Sugita *et al*., 1994). Aeromonads are known to be of great importance both economically and medically. The wide distribution of aeromonads in different aquatic ecosystems underlines their capacity to adapt to environments with different trophic levels. They are increasingly being reported as important pathogens for humans and for lower vertebrates, including amphibians, reptiles and fish (Janda and Abbott, 1998; Janda, 2001). Diseases caused by *A. hydrophila* (hemorrhagic septicemia, fin-tail rot, and epizootic ulcerative syndrome) have a major impact in aquacultures (Angka *et al*., 1995; Austin and Austin, 1999). At present, no vaccines for the protection of farmed fish against *A. hydrophila* infections are commercially available, although several studies have proved that various vaccine formulations may provide protection (Loghothiti *et al*., 1994; Karunasagar *et al*., 1997; Rahman *et al*., 2000; Chandran *et al*., 2002). Since *A. hydrophila* was first recognized as a significant opportunistic pathogen for humans, many efforts were dedicated to find methods for a correct identification (Pollard *et al*., 1990; O’Brien *et al*., 1994) and also for classification of species belonging to this genus. For many years, the taxonomy of *Aeromonas* spp. was confusing, and has undergone several significant revisions (Joseph and Carnahan, 1994). Popoff and coworkers (1981) placed *Aeromonas*
strains into DNA hybridization groups (HGs). Since then, the taxonomy of the genus has depended on a complex mixture of phenotypic and genotypic data. Actually, the genus *Aeromonas* contains at least 11 phenospecies and at least 14 HGs (Janda, 1991). The pathogenesis, pathogenic mechanism, and virulence factors responsible for selected *Aeromonas* infection in different species are not well understood.

The strains of *A. hydrophila* used in this study were isolated from freshwater, culture ponds, moribund Indian major carps and from aquarium fish. These isolates were identified to species level as described by Popoff & Lallier (1984). The phenotypic tests included: motility, presence of cytochrome oxidase enzyme, gas production from glucose, arginine, lysine and ornithine decarboxylase, VP-reaction, sensitivity to Vibriostat O/129, hydrolysis of aesculin, acid production from arabinose and salicilin and reaction in triple sugar iron (TSI). For further investigation four isolates i.e. Ah 65, Ah 68, Ah 70 and Ah 12a were used. These isolates were obtained from tail and fin rot affected Rohu (*Labeo rohitha*). Owing to their ubiquitous nature several *A. hydrophila* isolates were isolated from these samples. Strains isolated from the environment do not seem to differ from strains isolated from cases of infection with respect to the prevalence of virulence factors (Krovacek et al., 1994). However, it has been shown that certain virulent strains are more frequently isolated from patients with diarrhea as well as from diseased fish than from the environment (Kirov et al., 1994).

5.2. Polyacrylamide Gel Electrophoresis of Whole-cell Preparations of *A. hydrophila*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis has emerged as a useful tool in the classification and identification of bacteria. The whole-cell proteins of 10 environmental strains, 6 clinical strains of *A. hydrophila* and other *Aeromonas* spp viz; *A. sobria, A. caviae, A. trota, A. jandaei, A. veronii* and *A. schubertii*, were examined by
sodium dodecyl sulphate-polyacrylamide gel electrophoresis. For bacteria grown under standard conditions, electrophoresis defines protein banding patterns which can be considered as "finger prints" of the strain analyzed.

The conditions under which the bacteria are grown may have marked effects on the quality and quantity of proteins produced, some of which may be important in providing protective immunity and others may be important in virulence. Examples of growth conditions which affect such factors include the effect of growth medium on pilus production associated with virulence, the effect of temperature on virulence determinants, and the effect of culture age on the production of protective antigens. In addition, phenotypic differences have been detected by polyacrylamide gel electrophoresis between different serotypes of the same species and following bacterial passage of strains in vitro. As we were interested in defining protective immunogens for *A. hydrophila* we have attempted to define the whole cell proteins of the organism.

This study has shown the whole cell protein profiles of *A. hydrophila* to be similar on a broad view when the strains were grown under identical conditions and marked differences could not be detected when compared with other *Aeromonas* spp. and thus suggests that the technique would not be useful in distinguishing this species from other *Aeromonas* spp. The similarities of expressed proteins may be responsible for the degrees of cross-reactivity observed between *Aeromonas* spp. exposed to immune antiserum. On closer observation of the whole cell proteins profile of strains of *A. hydrophila*, it appears that there are minor variations in respect of the band numbers, intensity of bands and other properties of staining and electrophoretic mobilities. Similar findings were reported by Das *et al.* (2005). No attempt was made to define whether the profiles described were all protein, or whether some of the lines described were glycoproteins, lipoproteins or lipids.
Tabouret et al. (1992) concluded that 42 and 50 kDa bands appear to be common to all species of *Listeria monocytogenes* during SDS-PAGE of SDS extracted proteins of *L. monocytogenes*. On similar lines, from this study it can be concluded that among the several bands observed, bands of approximately 43kDa and 32kDa can be detected in all *A. hydrophila* strains.

A dendrogram of the cluster analysis based on protein profiles of the 16 strains of *A.hydrophila* is shown in Fig. 9. From, the dendrogram analysis, it was concluded that all the *A. hydrophila* strains could be grouped with 65% similarity while Das et al. (2005) deduced a similarity of 57% between different strains of the organism. They also deduced that isolates from related source of isolation showed higher percentage of relatedness. The dendrogram also revealed that all environmental isolates of the organism grouped into one cluster, except for 3 isolates namely Ah65, Ah41 and Ah71. The reason for this could be that these isolates are of clinical origin but entered the aquatic environment through sewage or run off from land. It could be summarized that *A.hydrophila* is a highly heterogeneous group and whole cell profile of the organism cannot be used as an ideal index for further characterization of this group. The identity of the large number of proteins described in whole cell preparations of *A. hydrophila* is unknown and is subject to investigation.

5.3. Outer membrane protein profile

OMP profile typing is a relatively simple and reliable typing technique which does not require any sophisticated equipment. The protein profiles of Sarkosyl-insoluble fractions from *A. hydrophila* isolates were similar with some differences. Homogeneity of OMP profiles within a species appears to be characteristic of members of the certain genus like *Bordetella* (Parton et al., 1975, Redhead et al., 1984). However in certain Gram-
negative bacteria, OMP profiles differ according to serotypes or biotypes (Barenkamp et al., 1981; Rapp et al., 1986).

The molecular weights of all the OMPs characterized ranged between 18 and 72 kDa. Several OMPs of A. hydrophila have been identified, cloned and characterized (Quinn et al., 1994; Lee et al., 1997; Nogueras et al., 1999; Fang et al., 2004; Tanuja et al., 2006; Khushiramani et al., 2007). A 43 kDa band was very prominent in all the strains. This OMP could be the major adhesin characterized by Fang et al. (2004). A similar band was also noticed in other Aeromonas spp. Rocha de souza et al. (2001) identified a 43 kDa adhesion in A. caviae. OMP pattern of A. hydrophila was different from the pattern obtain from Aeromoans spp. The difference /unique OMP patterns can either be a feature of A. hydrophila or it could be that the environment faced by these microorganisms play some role in the selection of strains with specific OMP patterns, the significance of which would require further investigation. This close homogeneity of the OMP patterns could also suggest a common clonal origin for these strains. Studies by other investigators have shown that OMP patterns correlate well with other indicators of clonal descendence (Ochman et al., 1984; Musser et al., 1987). The similarities among the protein profiles of both the whole-cell and detergent-soluble proteins indicate that common antigens are shared among these A. hydrophila strains.

OMP patterns have been used for typing strains of E. coli (Achtman et al., 1986), Shigella spp. (Valvano et al., 1991), Haemophilus influenzae (Barenkamp et al., 1981), Haemophilus pleuropulmoniae (Musser et al., 1987), Neisseria meningitidis (Olyhoek et al., 1987), Borrelia burgdorferi (Bundoc et al., 1989) and M. morganii (Senior et al., 1990). Further studies are required in this direction to use membrane typing as a method to
type strains of *A. hydrophila* and hence with this study we are unable to use this technique conclusively as a reliable typing method for *A. hydrophila*.

5.4. Cloning and expression of outer membrane protein of *A. hydrophila*.

A pivotal step in pathogenesis of virulent strains is the adhesion to and colonization of an appropriate host tissue or its associated components. The mechanisms underlying the adhesion of *Aeromonas* spp. to epithelial cells are not well understood and seem to be a complex process, apparently involving the occurrence of sequential or simultaneous factors (Merino *et al.*, 1996; Kirov *et al.*, 1999; Rabaan *et al.*, 2001; Gavin *et al.*, 2002).

Outer membrane proteins are immunologically important molecules in many Gram-negative bacteria. These proteins may affect the physiological functions of tissues and participate in mechanisms of pathogenicity, progression of infections and in development of inflammatory response (Lin *et al.*, 2002). In many inflammation processes caused by Gram-negative as well as Gram-positive bacteria, antibodies against bacterial proteins were found (Biswas *et al.*, 2000). These specific features of bacterial proteins (OMP) and their accessibility to the host immune system make them attractive as components of vaccines, convenient carriers for carbohydrate antigens and also as immunodiagnostic markers (Roy *et al.*, 1994).

As the causative agent of motile aeromonad septicaemia (MAS), *A. hydrophila* is of major economic importance to the fish farming industry. Vaccine development against this disease has special significance. However, due to the highly heterogeneous nature of *A. hydrophila*, it is impossible to use inactivated bacterial cells or LPS as a vaccine. Therefore, it is important to search for a conserved protective antigen to develop an effective vaccine. With this background, the present study was taken up to investigate the
immunological potentials of selected OMPs and their efficacy as useful vaccine candidates for protection against *A. hydrophila*.

Two OMPs were identified from the genome sequence available in the GenBank as vaccine candidates in the present study. These were OMP48 and OMPW.

### 5.4.1. Cloning and expression of *omp48*

In this study we report a novel outer membrane protein (OMP48) from a pathogenic strain of *A. hydrophila*. The primers designed based on the sequence of *omp48* gene of *A. veronii* amplified a 1305 bp fragment in *A. hydrophila*. This PCR product was cloned in TOPO 2.1 vector, sequenced by dideoxy termination method and the sequence was deposited in GenBank (acc. no. DQ177328). Nucleotide sequence analysis revealed that the gene possessed two ATG start codons. This is a common feature reported for bacterial transmembrane proteins with signal sequences where more than one N-terminal positively charged residues are present. In such cases it is most likely that the first ATG codon is the actual start codon of the gene (McIntyre *et al*., 1990). Considering the first ATG as the start codon, the gene had an open reading frame (ORF) of 1281 nucleotides with a capacity to encode a polypeptide of 426 amino acids with a protein of size being approximately 48 kDa. The gene also had a typical ribosome binding site 5’- AAGG –3’ (Shine Dalgarno sequence), 3 bp upstream of the first ATG initiation codon for methionine. The N-terminal sequence was hydrophobic and had 60.4% similarity to N-terminal sequence (GenBank acc no.AAB24934) of *A. hydrophila* outer membrane protein (Jeanteur *et al*., 1992). The predicted protein was acidic with a pI of 5.3 and had an estimated molecular weight of 46.89 kDa. The gene had G+C content 56%. In silico analysis revealed a signal sequence at position 1-25; a cleavage peptidase recognition site between positions 25-26 and several exposed antigenic regions for the protein. As expected
the protein sequence in this study possessed the characteristic features associated with signal sequence: an N-terminal hydrophobic region and the Ala-Phe-Ala cleavage recognition site at position 25-26 (Von Heijnen, 1985). A LamB-like outer-membrane protein was first identified in *A. veronii* by Vazquez-Juarez *et al* (2003). According to its N-terminal amino acid sequence, the 48-kDa protein was identified as OMP48. They also deduced that Anti-OMP48 antibodies in the rabbit serum significantly inhibited bacterial adhesion to HeLa cells by blocking OMP48 adhesive epitopes, while purified OMP48 showed a similar effect by competitive inhibition. Therefore, they concluded that OMP48 is a potential target antigen for vaccine development and induction of protective immunity through inhibition of host colonization. The omp48 gene isolated and sequenced from *A. hydrophila* was 97% identical to the nucleotide sequence and 98% identical to the translated amino acid sequence indicated by Vazquez-Juarez *et al* (2003).

The outer surface of *A. hydrophila* has been shown to be carbohydrate reactive (Quinn *et al*., 1994). Jeanteur *et al*. (1992) demonstrated the presence of an outer membrane protein in *A. hydrophila* which was a maltoporin, as its level increased when cells were grown in maltose-containing media and the channel it formed was blocked by maltose. Maltoporins (LamB proteins) are differentiated from other sugar porins by their 18 beta barrel strands which fold into trimeric structures, facilitating the diffusion of maltodextrins across outer membrane proteins (Wimley, 2002). The predicted 2D topology of the OMP48 sequence comprising of 18 beta strands and having similarities to other LamB porins in the Protein Data Bank clearly indicates the protein to be a maltoporin. A homologous protein porin I from *A. hydrophila* was also reported by Vazquez-Juarez *et al*. (2004). Cluster analysis shows that OMP48 of *A. hydrophila* is the closest to OMP48 of *A. veronii* and forms a separate group from other LamB porins described for *A. hydrophila*.
indicating that more than one type of LamB porin exists in the outer membrane of this bacterium. This is further reinstated from the lesser level of similarity (87%) it exhibits with the other maltoporin and LamB genes sequenced in *A. hydrophila*.

In this study the *omp48* was also detected in other *Aeromonas* sp viz; *A. jandaei*, *A. caviae*, *A. sobria* and *A. veronii*. This indicates that the gene is rather conserved among the genus. The presence of this OMP can also be the result of natural selection in the environment faced by these microorganisms, the significance of which would require further investigation. This OMP, isolated from a fish pathogenic *A. hydrophila* may be a conserved protein in the genus *Aeromonas* being present in serologically different *A. hydrophila*, *A. veronii*. The role of this protein in attaching and invading fish epithelial cells in vitro has been demonstrated (Vazquez-Juarez *et al*., 2003). Hence this gene is a suitable candidate antigen to provide broad spectrum protection.

It is likely that during the *Aeromonas* spp. colonization process, availability of maltose and maltodextrins (from nutrient digestion), might induce the expression of LamB-like proteins such as *omp48*. Therefore, *omp48* is a potential target antigen for vaccine development and induction of protective immunity through inhibition of host colonization. This is one of the few studies demonstrating the role of nonfimbrial proteins in *Aeromonas* spp. adhesion.

In this study, the *omp48* was cloned in expression vector after restriction digestion and therefore a truncated protein was expressed. However, the results show that the expression of this protein in pRSET B vector is efficient. There appears to be some background level expression in recombinant uninduced *E. coli* cells, but induction with IPTG clearly brought about over expression of the protein, the size being approximately 48
kDa. A similar pattern of expression was noticed in the *omp48* of *A. veronii* (Vazquez-Juarez *et al.*, 2003) and porin I of *A. hydrophila* (Jeanteur *et al.*, 1992). In addition, Omp48 of *A. veronii* was able to inhibit the adhesion of *A. hydrophila* and *A. caviae* (Vazquez-Juarez *et al.*, 2004).

The immunoassay results showed that the OMP48 protein is immunogenic in mice and rabbit. The Western blot data further reveals that the antibodies react with the whole cell protein of *E. tarda*, *E. coli*, *Salmonella* and *Vibrio* species. The deduced amino acid sequence of OMP48 showed similarity to LamB maltoporin. Therefore cross-reaction of antibodies to OMP48 with whole cell proteins of Gram-negative cultures used in this study is to be expected. However, it needs to be pointed out that the host *E. coli* BL21 strain used for cloning did not react with antibody in Western blot and *E. coli* MTCC 2939 showed 2 bands of different sizes (45 and 60 kDa) suggests that *E. coli* LamB proteins have different sizes. The cross reactivity was also observed with *E. tarda*, an intracellular fish pathogen in Western blot. There are no reports of LamB protein of *E. tarda*, but the data from Western blot in this study shows that *E. tarda* whole cell protein reacted in Western blot with antibodies against *A. hydrophila* OMP48 suggesting that *E. tarda* also express a LamB protein of similar size.

Use of outer membrane protein of *E. tarda* as vaccine has been reported by Kawai *et al.* (2004). With a 37 kDa outer membrane protein as vaccine of *E. tarda*, Kawai *et al.* (2004) obtained RPS value ranging from 50-70 with different strains of *E. tarda*. Similarly, studies with *A. veronii* have shown that the OMP48 is an adhesin with ability to bind to extracellular matrix proteins such as collagen, fibronectin, mucin and lactoferrin (Vazquez-Juarez *et al.*, 2004). Since the predicted *A. hydrophila* protein showed 98% similarity to *A. veronii* OMP48, it is to be expected that *A. hydrophila* protein also possibly
serves as an adhesin and plays a role in virulence of the organism. Vazquez et al. (2004) demonstrated that anti-OMP48 antibodies significantly inhibited the A. veronii adhesion to confluent HeLa cell monolayer and pretreatment of cells with purified OMP48 elicited competitive inhibition of adhesion. Therefore, it can be surmised that fish with high titer of antibody against A. hydrophila OMP48 might offer protection by inhibiting the initial step of invasion of this pathogen. Therefore, it can be concluded that OMP48 recombinant protein would be a valuable vaccine candidate. In addition, the observation that this protein has homology to outer membrane proteins of Vibrio spp, E. tarda and other members of Enterobacteriaceae, it may be suggested that this protein could offer protection to fish against infection from these pathogens.

5.4.2. Cloning and expression of ompW

In this study we cloned and expressed an outer membrane protein (OMPW) from a pathogenic strain of A. hydrophila. This gene was sequenced by the primers designed based on the sequence of ompW gene of the whole genome sequence of A. hydrophila ATCC 7966 (Seshadri et al., 2006). Outer membrane proteins are produced with an N-terminal signal sequence which directs the nascent polypeptide through the translocon in the inner membrane to the periplasmic space. The signal sequence is removed during translocation and the native protein is folded and inserted into the outer membrane (Bannwarth et al., 2003). Over expressed OMP without a signal sequence usually ends up forming inclusion bodies (Bannwarth et al., 2003). Hence, in the present study, the gene coding for ompW was amplified without the sequence of signal peptide. This protein was expressed in E. coli SG13009 competent cells using pQE-30 as cloning vector and expression was induced with 1mM IPTG.
The gene had an open reading frame (ORF) of 594 nucleotides with a capacity to encode a polypeptide of 196 amino acids with a protein of size being approximately 22kDa. The gene also had a typical ribosome binding site 5’- AAGG –3’ (Shine Dalgarno sequence), 3 bp upstream of the first ATG initiation codon for methionine. The N-terminal sequence was hydrophobic. The predicted protein was acidic with a pI of 5.1. The gene had G+C content 56%. In silico analysis revealed a signal sequence at position 1-24; a cleavage peptidase recognition site between positions 24-25 and several exposed antigenic regions for the protein. As expected the protein sequence in this study possessed the characteristic features associated with signal sequence: an N-terminal hydrophobic region and the Ala-Phe-Ala cleavage recognition site at position 24-25 (Von Heijnem, 1985). The nucleotide and amino acid sequence of ompW gene was observed to be 82% and 77% similar, respectively, to the sequence of ompW of A. hydrophila ATCC 7966 (Seshadri et al., 2006) as indicated by BLAST analysis.

Outer membrane proteins are often difficult to solubilize due to inclusion body formation. Therefore, native protein purification involves selective membrane extraction (Bannwarth and Schulz, 2003). Protein purification for the present study was performed by Guanidium hydrochloride denaturation method. Using pQE expression vector, the recombinant protein was obtained with N-terminal 6x histidine fusion proteins with high yields through single step purification. The expressed protein obtained was estimated to be approximately 24kDa by 10% SDS-PAGE. The recombinant protein was found to be highly immunogenic in rabbit and fish. The role of this protein in the pathogenesis of A. hydrophila is not yet known.

Lee et al. (1997) isolated the adhesins of A. hydrophila. From the major adhesin peak they isolated three OMPs of 43kDa, 28kDa and 22kDa. The 22kDa OMP extracted in
this study may be OMPW. Hence OMPW may have a role in the attachment of the organism on the host surface and establishing the disease. When rabbit polyclonal antibodies raised against recombinant protein was tested in Western blot with *A. hydrophila* and uninduced and induced nonrecombinant *E. coli*. reaction was observed only with *A. hydrophila* while non-recombinant *E. coli* were negative. This proves that the protein expressed was of *Aeromonas* origin and antigenicity of the protein is maintained.

When tested by ELISA, unimmunized fish had low background titers (1:8) against this protein of *A. hydrophila*. This could be due to exposure of fish to *A. hydrophila* which is a common inhabitant of the aquatic environment. On day 14, after primary immunization and one booster, ELISA titers were >4000. On day 28, the ELISA titer rose to 1:32,000. The results suggest that *omp W* is highly immunogenic in fish. The protein expressed in the present study shows promise as vaccine candidate in aquaculture and could probably be used for the protection of cultivated Indian major carps.

**VI. SUMMARY**

*Aeromonas* species are known to be of great importance both economically and medically. They form a complex group of ubiquitous Gram-negative, facultatively anaerobic, motile, water borne bacteria that are widely isolated from clinical, environmental and food samples and are considered opportunistic human pathogens and